GLYOXALINES IN URINE AND THE KNOOP TEST

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A thesis submitted in conformity with the requirements for the degree of Master of Science by the University of Alberta.

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University of Alberta
1943

ACKNOWLEDGEMENT

The writer gratefully acknowledges her indebtedness to Professor G. Hunter for his invaluable guidance and assistance throughout the progress of the investigation.

Her thanks are due Dr. M. M. Cantor for helpful advice, valuable criticism and co-operation in obtaining material from the Department of Obstetrics and Gynaecology.

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I INTRODUCTION

In 1908 Engeland (7) first proved the presence of glyoxaline derivatives in human urine when he isolated from it histidine and two other unidentified glyoxalines. Histidine has subsequently been isolated from measles urine by Hunter (10) and from pregnancy urine by Armstrong and Walker (2). Ackermann and Fuchs (1) and Hunter and Raragosky (unpublished data) succeeded in isolating histidine from normal urine.

Histamine too has been isolated but in very small quantities. Ackermann and Fuchs (1) obtained only 0.9 mg. of Histamine Dipicrate from 1000 litres of normal urine. Recently Kapeller - Adler (18) isolated a small amount from urines of pregnant women with eclampsia. Thus it is unlikely that histamine constitutes a significant part of the glyoxalines present in urine.

In recent years interest in urinary glyoxalines has been increased by the claims notably of Kapeller - Adler (16), (17) that histidine excretion is increased during pregnancy. She

claims that the excretion of histidine in the urine is dependent on the presence of an enzyme, histidinase, in the liver. Her theory is that, in pregnancy, the histidinase activity of the liver is inhibited and hence the appearance of unchanged histidine in the urine. She bases her evidence largely on the finding that the urines of pregnant women give a positive Knoop test that is not found in the urine of men or of non-pregnant women.

In view of the facts that histidine has been isolated from normal male and female urine, and the Knoop test is described as being specific for histidine, there appears to be some discrepancy in Kapeller - Adler's theory.

The present investigation was carried out to study the relationship that does exist between urine glyoxalines and the Knoop test. During the course of our experiments we found that there are several substances, even in normal urine which give a Knoop test. Among these is histidine. But the bulk of the colour given in the test appeared to come from material other than histidine and not glyoxaline in composition. The investigation then

proceeded with a view to determine the nature of the main Knoop giving substance.

II HISTORY OF THE KNOOP REACTION

When a very slight excess of bromine is added to a solution of histidine, and the solution then warmed, a wine - mauve colour is produced, the intensity of which depends on the concentration of histidine present. This is the Knoop test as it was first described in 1908 (20). The test was said to be specific for histidine and was sensitive in a dilution of 1:1000. Histamine also gave a reaction but was much less sensitive.

Hunter (11) modified the reaction somewhat by using chloroform to remove the excess bromine and thereby increased both the delicacy and certainty of the test. The sensitivity was now increased to 1:10,000.

In 1932 Armstrong and Walker (2) found the reaction worked best in a faintly acid solution.

Kapeller - Adler (15) claimed to further sensitize the reaction so as to give a test in a dilution of 1:50,000. She added a 1% bromine in 33% acetic acid solution in slight excess, let it stand for ten minutes, and then made the solution ammoniacal with an ammonium hydroxide - ammonium carbonate mixture. On heating in a water bath for five minutes a blue - violet colour developed.

Conrad and Berg (5) recommended the use of arsenious oxide for the removal of excess bromine.

Recently Racker (22) claimed to increase the sensitivity of the Knoop test to 1:100,000 by the presence of sodium urate.

The Knoop test is not quite specific for histidine. It is also given by histamine or iminazolylethylamine (Knoop (20) and Hunter (11)) and by N - methylhistidine, where the methyl group is attached to the N of the ring, (Linneweh et al (21) and Armstrong and Walker (2)). Adrenaline and tyramine also give a reaction with bromine but of a different type, and easily distinguishable from the histidine test. (Armstrong and Walker (2)). Tryptophane (Hunter (11)) gives a reaction in the cold and the colour developed is easily extractable with amyl alcohol. Indoxyl gives a bright red colour which is readily extractable with amyl alcohol.

The test is interfered with by tyrosine, tryptophane, methioneine (Woolley and Peterson (34)) and glycine (Kapeller - Adler (16)).

Attempts have been made by many workers to apply the Knoop test to human urine especially since Voge (26) suggested it as a means for the early detection of pregnancy. He claimed 95%

agreement with Ascheim-Zondek tests. Burt - White (4) obtained similar results.

Many contradictory results have been published since then about histidine excretion in urine. Kapeller - Adler (16), (17), (18) has written a series of papers supporting her claim that a positive Knoop test is given only by urines of pregnant women. Papers by Weiss (33), Renton (23) and Dello Jojo (6) upheld Kapeller - Adler's theory.

However Földes (8) seriously questioned the value of the pregnancy test since he obtained tests in urines of non pregnant women. Also Tschopp and Tschopp (24) came to the conclusion that histidine is excreted sometimes in normal but more frequently in some pathological conditions. This was especially so in liver diseases and allergic conditions.

It is apparent that the literature on the Knoop test is full of variations and contradictions and at the present time no uniform agreement exists on the specificity of the Knoop test. Thus it is evident that there is need for a systematic study of the Knoop reaction.

III SCOPE OF THE WORK

The present study has resolved itself into three main topics:

A. The development of a satisfactory procedure for the Knoop test.

B. A study of Knoop test in relation to urinary glyoxalines.

C. A study of the nature of the Knoop giving substances in human urine.

IV EXPERIMENTAL

A. The Development of a Satisfactory Procedure for the Knoop Test

The reagents and method used were similar in part to those employed by Conrad and Berg (5). We did, however, introduce certain modifications.

Reagents Used

1. Histidine solution - concentration 1 mg. per ml.
2. 5N Acetic Acid.
3. Saturated bromine water.
4. Saturated solution of arsenious oxide in 10% ammonia.
5. Ammonium acetate solution made by just neutralizing concentrated ammonia with glacial acetic acid.

In developing the procedure we found there were several variable factors in the production of the maximum colour.

These factors were:-

(a) reaction of the test solution.

(b) time of bromination.

(c) large excess of bromine.

(d) temperature during bromination.

(e) volume of solution.

(f) ammoniacal mixture used.

(a) Reaction of the test solution.

Three tubes containing 0.1 ml. histidine solution and varying amounts of acid

(1) no acid

(2) 2 drops 5N acetic

(3) 2 drops glacial acetic

Bromine was added drop by drop until an excess was present. The tube was let stand for 5 minutes, after which the excess bromine was removed by the addition of a drop of the arsenious oxide solution. Finally 0.5 ml. of ammonium acetate solution was added and the tubes set in a boiling water bath.

There was only a trace of colour in (1) but about the same good purple colours in (2) and (3).

This shows that we must brominate in an acid solution.

(b) Time of bromination.

Four tubes - each containing 0.1 ml. histidine solution, 2 drops 5N acetic and 1 drop (excess) bromine.

After 2, 4, 6 and 10 minutes we added 1 drop arsenious oxide solution and 0.5 ml. ammonium acetate and heated for 5 minutes in a boiling water bath. The tube with only 2 minutes bromination

was lightest in colour, the others all had much the same colour. This shows there is a time factor at this stage.

Five minutes was taken as a suitable time for bromination.

(c) Effect of a large excess of bromine.

Three tubes - each containing 0.1 ml. histidine solution and 2 drops 5N acetic acid.

(1) added 3 drops bromine

(2) added 3 drops bromine after 5 minutes

(3) added 3 drops bromine after 9 minutes

After ten minutes the Knoop tests were completed. Number (3) was very faint, (1) and (2), however, were about the same. This shows that a large excess of bromine does not give less colour development as long as the excess is removed before the addition of the ammoniacal solution. However, a large excess still does not dispense with the need for 5 - 10 minutes contact with bromine. On the other hand, the solution must be fully saturated with bromine in order to get the maximum Knoop.

(d) Temperature during bromination.

Five tubes containing 0.1 ml. histidine solution and 2 drops 5N acetic acid left for 10 minutes with excess bromine

(1) in frigidaire (about 4 - 5°C)

(2) at 24°C

(3) at 37°C

(4) at 55°C

(5) at 80°C

Knoop on (5) was quite negative and there was only a faint colour in (1). Good colours in (2), (3) and (4) - if anything, it is best at 37°C.

Thus it would appear that 5 minutes at 37°C represents good conditions for a maximum Knoop.

(e) Volume of solution.

Tests showed that the smaller the volume in which the colour is produced the greater the final colour.

(f) Ammoniacal mixture used.

Tests were done using

(1) saturated ammonium sulphate solution.

(2) ammonium hydroxide - ammonium carbonate mixture as used by Kapeller - Adler (15).

(3) ammonium acetate solution made by just neutralizing concentrated ammonium hydroxide with glacial acetic acid.

From the foregoing tests the optimum conditions were chosen and incorporated into our procedure for the Knoop test.

Standard Procedure for doing the Knoop Test.

1. To 0.1 ml. of histidine solution in a test tube is added two drops of 5N acetic acid and saturated bromine water drop by drop until there is a definite excess (usually 1 - 2 drops are sufficient).

2. The tube is set in a water bath at 37°C for 5 minutes.

3. Excess bromine is removed by the addition of a drop of arsenious oxide solution, immediately after which 0.5 ml. of ammonium acetate solution is added.

4. The test tube is placed in a boiling water bath for 5 minutes for full color development.

Sensitivity of Our Method vs. Kapeller - Adler's Method.

Two series (A and B) of tubes containing 0.1, 0.08, 0.06, 0.04, 0.02 and 0.005 mg. histidine in 0.1 ml. of solution.

Final volume of test solution is about 1.0ml.

Series A. - Our Method.

0.06 tube showed a definite colour

0.04 tube was questionable

Taking 0.05 mg. in 1 ml. of solution as the limiting concentration, the sensitivity of the test

is 1:20,000.

Series B. - Kapeller - Adler's Method (15)

0.1 tube showed a colour about half as strong as same tube in series A.

0.06 tube showed no colour.

Therefore, sensitivity by this method is about 1:12,500.

We have thus been unable to duplicate Kapeller - Adler's claim that her test is sensitive in a dilution of 1:50,000.

It might be mentioned here that we investigated Racker's claim (22) of increasing the sensitivity to 1:100,000 by the presence of sodium urate but we found a discrepancy in the method. Our tests showed that both sodium urate and uric acid themselves gave a bright pink colour in the Knoop test, the intensity of which was roughly proportional to the amount used. We concluded, therefore, that the increased colour obtained was derivable from the oxidation product of uric acid in contact with bromine, namely alloxantin. Alloxantin gives a pink colour under the conditions of the Knoop test even without bromination. Alloxan and parabanic acid both gave positive tests as well.

Comparison of Knoop Test on Histidine and Histamine.

Both the histidine and histamine solutions used had a concentration of 1 mg./ml.

	Colour of Knoop Test
1. 0.1 ml. histidine	mauve colour
2. 0.2 ml. histidine	deep purple
3. 0.1 ml. histamine	pale yellow
4. 0.2 ml. histamine	darker yellow

In more concentrated solutions histamine gives a purple colour. Its sensitivity is only about a third that of histidine.

B. The Knoop Test in Relation to Urinary Glyoxalines.

After the development of a satisfactory method for the test, we undertook to study the Knoop test of urine. Assuming that histidine was responsible for the Knoop test, we attempted to correlate the Knoop test with the glyoxaline content of the urine as determined by the diazo method of Hunter and Raragosky (14).

Preparation of the Glyoxaline Fraction from Urine

Five ml. of urine is placed in a 15 ml. centrifuge tube, followed by 4.0 ml. of 40% normal lead acetate with shaking. Four ml. of 2.0 N sodium hydroxide is then added, and the contents thoroughly mixed. The tube is then centrifuged until the precipitate is well packed and the supernatant is poured off through a small filter paper into a 50 ml. centrifuge tube. The drained lead precipitate is washed well with 3.0 ml. of water and the tube again centrifuged. The supernatant fluid is filtered as before. The filtrate, which is clear, colourless and slightly alkaline, contains a trace of lead. This lead is precipitated by the addition of a few drops of 20% disodium hydrogen phosphate solution. The tube is again centrifuged and the supernatant is poured into a glass evaporating dish which is then placed on a

boiling water bath and the contents boiled to dryness. The white crystalline residue is dissolved in a little water, transferred to a graduated centrifuge tube and made up to 10 ml. The glyoxaline fraction has thus twice the volume of the native urine.

The Diazo Test.

The reagents used

1. Diazo Reagent - Koessler and Hanke (19)
   - made from two stock solutions.
   A. Nine gm. of sulphanilic acid with 90 ml. of 37% hydrochloric acid is made up to 1 litre with water.
   B. Fifty gm. of 90% sodium nitrite is made up to 1 litre with water. Solution is kept in a refrigerator.

   The reagent is prepared by placing in a 50 ml. volumetric flask, immersed in ice water, 1.5 ml. of solution A along with 1.5 ml. of solution B. After five minutes 6.0 ml. of solution B is added, and after 5 minutes more the flask is filled to the 50 ml. mark with cold water. The glass stopper is inserted and the contents thoroughly mixed. After 15 minutes the

reagent is ready for use.

2. A freshly prepared 1.1% solution of best quality anhydrous sodium carbonate.

The colour standard of Hunter (12) was used. This is made from two stock solutions.

A. Stock methyl orange - consists of 0.1 gm. of pure methyl orange made up to 100 ml. with water.

B. Stock congo red - consists of 0.2 gm. of pure congo red made up to 100 ml. with water.

To about 80 ml. of distilled water in a 100 ml. volumetric flask is added 0.40 ml. of stock congo red solution and 0.06 ml. of stock methyl orange solution. The contents are mixed and made up to volume. Some of this colour standard is placed in the right hand cup of a semi-micro colorimeter of the Duboscq type.

Into the other cup is placed (0.5 - x) ml. of water, 2.5 ml. of 1.1% sodium carbonate solution and 1.0 ml. of diazo reagent, and the time noted. The cup is agitated to mix. One minute later x ml. of the urinary glyoxaline fraction is added. The cup is quickly placed in the colorimeter and the contents mixed by raising and lowering the plunger several times. The plunger is then set at

a depth of 10 mm. The standard solution plunger is moved to match. Maximum colour is reached after 3 - 4 minutes.

All determinations are carried out with at least two quantities of the solution under examination in order to test the proportionality.

With the test solution set at 10 mm. 1 mm. of standard is equivalent to 0.001352 mg. of histidine or 0.0010 mg. of "glyoxaline."

Knoop tests were done both on the native urine and on the glyoxaline fraction (filtrate from lead precipitate). In order to compare the Knoop tests on the two, the native urine was diluted once with water.

Thus, since 1.0 ml. of the glyoxaline fraction was used for a test, 0.5 ml. of urine was diluted with 0.5 ml. of water for the corresponding test. The tests were carried out as previously described.

The degree of the test was indicated relatively

trace - pale yellow
\+ - pale violet
++ - mauve colour
+++ - dark mauve to purple.
++++ - very dark purple.

The determinations were done on pregnancy urines both pre and post delivery. The pre delivery

sample was a single specimen usually taken about 6 to 12 hours before delivery where this was possible. However, the post delivery sample was a 24 hour specimen collected about the third day post partum. The results are tabulated.

Subject	Pre or Post	Sp. Gr.	Glyoxaline Content mg./5 ml.	Knoop Test Nat.	Knoop Test Glyoxaline Fraction
1.	Pre	1.0170	2.60	+++	+
	Post	1.0076	1.41	+	-
2.	Pre	1.0130	.75	+	-
	Post	1.0097	.76	++	+
3.	Pre	1.0095	.35	+	+
	Post	1.0142	1.05	+	-
4.	Pre	1.0092	.48	+	+
	Post	1.0100	.30	++	-
5.	Pre	1.0063	.28	+	-
	Post	1.0063	.26	+	-
6.	Pre	1.0235	1.26		
	Post	1.0121	1.11	++	+
7.	Pre	1.0111	.96		
	Post	1.0069	.97	+	+
8.	Pre	1.0126	.75		
	Post	1.0059	.62	+	-
9.	Pre	1.0092	.65	+	-

Subject	Pre or Post	Sp. Gr.	Glyoxaline Content mg./5 ml.	Knoop Test	
				Nat.	Glyoxaline Fraction
10.	Pre	1.0073	.41	+	-
11.	Pre	1.0054	.22	+	-
	Post	1.0085	.70	+	Trace
12.	Pre	1.0275	2.70	++++	+
	Post	1.0116	1.34	++	Trace
13.	Pre	1.0125	1.16	+	-
	Post	1.0108	.90	+	-
14.	Pre	1.0079	1.26	+	Trace
	Post	1.0124	1.70	+++	Trace
15.	Pre	1.0293	1.70	+	+
	Post	1.0087	.50	+	-
16.	Pre	1.0095	.85	++	Trace
	Post	1.0122	.88	+	Trace
17.	Pre	1.0067	.20	+	-
	Post	1.0106	.44	+	-
18.	Pre	1.0090	.50	+	-
	Post	1.0106	.75	+	-

There is no proportionality whatsoever between the glyoxaline content and the Knoop test.

In the case of Subject 2, the glyoxaline contents per 5 ml. of the pre and post specimens were practically identical, yet the Knoop tests on both the native urine and glyoxaline fractions are greater in the case of the post specimen.

In the glyoxaline fraction a "one plus" Knoop test is given by specimens in which the glyoxaline content varies from 0.35 - 2.70 mg./5ml. and a negative test is given by a specimen with a content of 1.41 mg./5 ml.

In the native urine, too, a "one plus" test is given by specimens in which the content varies from 0.20 - 1.70 mg./5 ml. and yet a specimen with only 0.30 mg./5 ml. gave a "two plus" Knoop. Two specimens with 1.70 and 2.60 mg./5 ml. gave "three plus" Knoop tests and one with 2.70 mg./5 ml.gave a "four plus" test.

However, in practically every case, there is a marked difference between the Knoop tests of the native urine and the glyoxaline fraction.

This could mean one or both of two things.

(1) The glyoxaline fraction contains substances which inhibit Knoop colour development.

(2) Some Knoop giving substance is removed in the

lead precipitate.

(1) On investigating the dependability of the Knoop test on the lead filtrate it was found that urines vary in their behaviour.

When 0.1 or 0.2 mg. histidine per ml. is added to the filtrates and a Knoop test done, those with histidine added are often questionably distinguishable from filtrates without added histidine.

If 1.0 or 2.0 mg. histidine per ml. is added, we get a slight brown intensification.

Some lead filtrates will, however, give good tests and, on the addition of histidine, will show an intensification of colour approximating the histidine added.

(2) The lead precipitate from the preparation of the Glyoxaline Fraction was washed and then freed with 5 N sulphuric acid until the supernatant was acid to congo red paper. The supernatant was centrifuged off and a Knoop test done on it gave a strong brown colour. A little of this colour was extractable with amyl alcohol. A diazo test on this supernatant was a pale yellow colour.

Thus, we have evidence that one or more Knoop giving substances are removed in the lead precipitate.

These two experiments explain the difference in the Knoop test of the Native Urine and the Glyoxaline Fraction.

C. A Study of the Nature of the Knoop Giving Substances.

After the demonstration of the presence of Knoop giving substances in the lead precipitate, we undertook to study the nature of these substances.

We attempted to isolate these substances but were unsuccessful in doing so. We did, however, fractionate the precipitate so that we obtained a sticky resinous like concentrate which gave a strong Knoop test.

In the lead precipitate there are present lead salts of such inorganic radicals as, phosphates, carbonates, sulphates and sulphide; such organic materials as sugars, acetone bodies, phenols and purines; and such pigments as urochrome, urobilin and its chromogen and bilirubin.

The lead precipitate was fractionated into two portions:

A. That which comes down on the addition of normal lead acetate to the urine - Acid Lead Fraction.

B. That which comes down on the addition of sodium hydroxide to the filtrate of the acid lead fraction - Basic Lead Fraction.

Lead Fractionation

To one litre of urine in a 3 litre erlenmeyer flask was added half its volume (500 ml.) of 40% normal lead acetate solution. The fine light buff coloured precipitate was allowed to settle for a few hours, after which the clear yellow supernatant was carefully poured off through a dry filter paper so as to remove floating particles. The remainder of the mixture was centrifuged in 250 ml. cups and the supernatant added to the filtrate.

The precipitate (Fraction A) was washed well by dispersing it thoroughly in distilled water and centrifuging the mixture again. The wash water was discarded.

To the filtrate from the acid lead precipitate was then added 500 ml. of 2.0 N sodium hydroxide. The creamy coloured precipitate was allowed to settle, and the supernatant - the glyoxaline fraction, which is almost colourless, was here discarded.

The precipitate (Fraction B) was collected in a 250 ml. centrifuge cup and washed as before.

Both precipitates were now treated in exactly the same way.

The precipitate was transferred to a mortar and then 5 N sulphuric acid was added in small quantities

until the supernatant was blue to congo red paper. Then the acid was added two drops at a time until the supernatant gave a positive test for sulphate ion with barium chloride. The mixture was centrifuged and the clear yellow-orange coloured supernatant poured through a dry filter paper.

Knoop tests were done on an aliquot of each of the supernatants. In all but two of the urines investigated, there was a greater concentration of Knoop giving substances in the basic fraction.

The acid lead fraction was more orange in colour than the clear golden yellow colour of the basic fraction. The precipitate left, after the removal of the acidic and basic fractions was supposedly lead sulphate. If, however, more acid was added to this precipitate, a dark orange-brown supernatant was obtained which did give a Knoop test. On standing, it turned even darker and eventually blackened, thus indicating decomposition had taken place.

It was decided to devote the investigation mainly to the basic lead fraction since it contained the greatest portion of the Knoop giving substance. The acid lead precipitate was washed with acetone, dried and stored as a dry powder for future investigation.

The lead free supernatant from the basic fraction was left overnight in the refrigerator. Small brown crystals separated out. These were filtered off and

on testing proved to be uric acid.

The filtrate was concentrated in vacuo. At a certain stage the solution became cloudy and a precipitate came down. It was filtered off through a sintered glass funnel. The Knoop test was negative and it was discarded.

Mercury Fractionation

The filtrate was then put through a mercury fractionation. Mercuric acetate (25%) was added until there was no further precipitation. The solution was then made just alkaline to litmus with sodium carbonate (10%) and the mixture centrifuged. The precipitate was washed well with a little distilled water containing a few drops of sodium carbonate. The precipitate was suspended in a little water, made acid with a little sulphuric acid and then hydrogen sulphide was bubbled through to free the material of mercury. The mercuric sulphide was centrifuged off, washed and discarded, while the filtrate and washings were evaporated in vacuo to remove the hydrogen sulphide. A Knoop test done on the concentrate showed that there had been very little loss of material in this fractionation.

Silver Fractionation

The procedure used was similar to that described by Vickery and Leavenworth (25).

One ml. of the concentrated filtrate from the mercury fractionation was put through a silver fractionation.

Silver nitrate (10%) was added until a definite excess was present as was demonstrable by the development of an immediate brown spot - test with barium hydroxide solution. The precipitate (A) was centrifuged off and washed. Saturated barium hydroxide solution was added to the filtrate until the solution had reached a pH of 6.4. Precipitate (B) was centrifuged off and washed. To this filtrate more barium hydroxide was added until the solution now was at a pH of 7.4. Precipitate (C) was centrifuged off and washed. Finally barium hydroxide was added until there was no further precipitation. Precipitate (D) was centrifuged off and washed. Filtrate (E) was saved.

Precipitates A, B, C, D and filtrate E were made acid with sulphuric acid and freed with hydrogen sulphide. The supernatants were centrifuged off and evaporated to dryness on a boiling water bath. The residues were dissolved in a little water and made up to a volume of 1 ml.

Knoop and diazo tests were done on the five solutions.

Solution	Knoop Test	Diazo Test
A.	negative	negative
B.	negative	negative
C.(6.4 - 7.4)	++	faint pink
D.	negative	negative
E.(filtrate)	faint yellow	faint yellow

There seemed to be a little loss of the Knoop substance in this fractionation. However, the remainder of the concentrate from the mercury fractionation was put through the silver fractionation. Only the precipitate between the pH range of 6.2 - 7.6 was saved. The precipitate was washed and freed in the usual manner with hydrogen sulphide. The supernatant was evaporated to dryness on a water bath and made up to 10 ml. volume. Knoop test was positive but showed a little loss in the fractionation.

Phosphotungstic Acid Precipitation

The method of Hunter and Eagles (13) was followed. The solution from the silver fractionation was made 0.5 N with respect to sulphuric acid by the addition of the requisite amount of 10 N acid. Then a 20% solution of phosphotungstic acid in 0.5 N sulphuric

acid was added to a point where further addition caused no precipitation in a test portion of the supernatant. The tube was lightly stoppered and allowed to stand overnight in the refrigerator for complete precipitation to take place. In the morning the precipitate was centrifuged off care being taken to keep the temperature of the solution as low as possible throughout the entire procedure. The precipitate was washed twice with its own volume of ice - cold 0.5 N sulphuric acid to which had been added a little of the phosphotungstic acid solution.

Solid barium hydroxide was added to the precipitate until the supernatant solution tested definitely alkaline to litmus. The mixture was centrifuged and the filtrate decanted through a filter. The precipitate was washed thoroughly three times with 0.1 N barium hydroxide solution and the washings added to the filtrate

The strongly alkaline filtrate and washings were heated to boiling. 5 N sulphuric acid was added till no further precipitation occurred. The barium sulphate precipitate was centrifuged off and washed with a little hot water. Quantitative removal of both Ba^{++} and $SO_4^{=}$ ions was effected by the addition of the required volumes of 0.1 N barium hydroxide and sulphuric acid.

Knoop and diazo tests were done on the phosphotungstic acid precipitate and filtrate fractions.

	Knoop	Diazo
Precipitate fraction	negative	negative
Filtrate fraction	positive	pale pink

Thus the Knoop giving substance is not precipitated by phosphotungstic acid.

The filtrate fraction was concentrated in vacuo to a small volume and a little absolute ethanol added to bring about crystallization. The tube was stoppered and set in the refrigerator over night. In the morning there was no sign of crystals. The alcohol and as much of the water as possible were evaporated in vacuo. The material left in the tube was a sticky mass. It was taken up in a little absolute methanol and left in the refrigerator for crystallization. The material, in the morning, showed no sign of crystal structure and had darkened considerably indicating decomposition had taken place and was discarded.

The foregoing fractionation procedures were repeated twice more. The first time the material was lost in the silver fractionation and the second time the Knoop giving substances were unstable to the mercury fractionation.

No reason for these inconsistent results could be discovered.

The Potassium Salt Formation Method of Extraction.

This procedure was found to be the most stable method of extracting the Knoop giving substance.

2.0 N potassium hydroxide was used as the base in the basic lead precipitation.

The basic lead precipitate was treated with 5 N sulphuric acid until the first faint blue tinge with congo red paper (pH about 3.5 - 4) was obtained. At this pH it was thought that all of the lead salt of the Knoop giving substance was soluble and the supernatant solution was centrifuged off. The precipitate left was found to contain some Knoop substance but the loss of this amount was more than compensated for by the stability of the extract.

The supernatant was treated with a saturated solution of potassium sulphate. The theory was that the potassium ion replaced the lead ion in the salt of the unknown substance and the lead was removed as insoluble lead sulphate. Potassium hydroxide was used as the base above in order to prevent the formation of mixed sodium - potassium salts.

The lead sulphate was filtered off and the filt-

rate was concentrated in vacuo to a small volume and set in a vacuum desiccator to dry. After several days the dry residue was extracted with absolute methanol, reconcentrated and dried again.

In all, five - 2 litre lots of urine were treated in this way. Even after standing in a vacuum desiccator for periods as long as 2-3 weeks the yellow brown residues showed no sign of crystals but remained as a sticky mass. The material was stable, however, and showed no sign of darkening. One of the fractions did become hard and resinous like but not crystalline. The Knoop test also was stable - showed no sign of becoming weaker.

The material was readily soluble in water and methanol forming a yellow brown solution, but not so soluble in ethanol. A sodium fusion showed the absence of sulphur, phosphorus and halogens but gave a positive test for nitrogen.

The material decolorized bromine and alkaline potassium permanganate and also gave a reddish colour with ferric chloride. The diazo test was weak yellow colour. The material gave a positive Ninhydrin test. This was rather surprising but it could have been due to the presence of an impurity. The material gave a negative biuret, Millon's, Tollen's (ammoniacal

silver nitrate), glyoxylic and phenylhydrazine tests.

Further Notes on Procedure.

Barium hydroxide and concentrated ammonia were used in some of the fractionations as the base for the precipitation of the basic lead fraction but were found to be not as suitable as either sodium or potassium hydroxide. The precipitate was more granular in type but the filtrate still contained some colour.

Hydrogen sulphide was used in several of the fractions to free the lead precipitate. The method was quite satisfactory if the hydrogen sulphide were removed as soon as possible and the solution made neutral to litmus with potassium hydroxide.

Solvent Extraction Method

Several solvent extractions of the basic lead aqueous concentrates were tried.

Benzene, toluene, ether, acetone, ethyl methyl ketone and chloroform failed to extract anything from the aqueous solution. Both isoamyl and n-butyl alcohol extracted some of the material, the latter one being the better of the two.

A procedure, similar to the Bogomolow (3) test for urobilin was tried.

The aqueous solution was made slightly acid with glacial acetic acid and a few drops of copper sulphate (5%) were added. The mixture was shaken with butyl alcohol and centrifuged to aid in the separation of the phases. Two layers formed:

(a) a green water layer

(b) a pink - brown alcohol layer.

The alcohol layer was drawn off and evaporated in vacuo as far as possible. The residue was taken up in a little water and the Knoop test done on it was positive.

The water layer was taken to dryness and the residue taken up in a little water. Knoop test on this fraction was negative.

It was thought that the copper formed a salt with the material which was soluble in butyl alcohol. An attempt was made to crystallize this copper salt but it was unsuccessful.

Garrod's method (9) of saturating the urine with ammonium sulphate and extracting the yellow pigment from the ammonium sulphate solution with absolute alcohol was modified.

The basic lead fraction was saturated with anhydrous sodium sulphate. On adding absolute methanol an insoluble residue was thrown down and two layers

were formed:

(a) water layer

(b) alcohol layer containing the yellow colour and the Knoop giving substance.

The alcohol layer was separated in a separatory funnel, drawn off, evaporated in vacuo to a small volume and then dried in a vacuum desiccator over night. The dried residue was extracted with absolute methanol. This alcohol extract gave a strong Knoop test. It was again evaporated to a small volume and left in the desiccator to crystallize. Even after a week the material remained sticky and there was no sign of crystals.

Throughout all our experiments it was noticed that the intensity of the Knoop test was directly proportional to the intensity of colour of the test solution. This suggested that the Knoop giving substance was a pigment in the urine. In many ways it resembles Weiss' (27),(28),(29),(30),(31),(32) urochromogen.

Like urochromogen, it is found in the basic lead precipitate, it has acid properties since it forms salts soluble in water and alcohol but insoluble in ether, and it reduces permanganate. Also it gives negative Millon's, glyoxylic acid tests.

Unlike urochromogen, it gives a red colour with ferric chloride (Weiss says urochromogen gives a green colour) and it fails to reduce ammoniacal silver nitrate. The diazo test is weak.

Other Knoop Giving Substances in Urine.

In addition to histidine, indoxyl, and the substance in the basic lead precipitate we have evidence that there are at least three other Knoop giving substances in urine.

The acid lead fraction contains two such substances:

(a) one which is precipitated by barium hydroxide

(b) one which is left in the filtrate from the barium hydroxide precipitation.

A third Knoop giving substance was found in the filtrate from the mercury fractionation of the Glyoxaline Fraction of the urine.

These three have not been investigated as yet.

V SUMMARY

1. A method for doing the Knoop test has been described.
2. Histidine is not the only substance in urine giving a positive Knoop test but evidence points to at least five other substances.
   (a) Two in the acid lead fraction
   (b) One in the basic lead fraction
   (c) One in the filtrate from the mercury fractionation of the Glyoxaline Fraction of the urine.
   (d) Indoxyl
3. A resinous like concentrate of the substance in the basic lead fraction was obtained.

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